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Original Article

Laminin 511 E8 fragment promotes to form basement membrane-like structure in human skin equivalents

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ABSTRACT

Introduction: Laminin 511 (LM511), a component of the skin basement membrane (BM), is known to enhance the adhesion of some cell types and it has been reported to affect cell behavior. A recombinant fragment consisting of the integrin recognition site; E8 region of LM511 (511E8) has also been studied. 511E8 has been reported by many as a superior culture substrate. However, the effects of 511E8 on human skin cells remain unclear. In this study, we added 511E8 during the culture period of a reconstituted skin equivalent (SE) and investigated its effect on the formation of BM-like structures.
Methods: SEs were formed by air-liquid culture of human foreskin keratinocytes (HFKs) on contracted type I collagen (Col-I) gels containing human fibroblasts. We compared the BM-like structures formed with and without 511E8 during HFKs culture periods. Morphological analysis, gene expression analysis of extracellular matrix components, and localization analysis of 511E8 in order to identify where 511E8 works were performed.
Results: Immunohistochemical observation by light microscopy showed an accumulation of BM components between the gels and cell layers regardless of the addition of 511E8. There was a stronger and more continuous positive staining for LM α3, type IV collagen, and type VII collagen in the 511E8-added group compared to the no-added group. Transmission electron microscopic observation showed that the continuity of BM-like structures was increased with the addition of 511E8. Furthermore, gene expression analysis showed that the 511E8 addition increased some BM component genes expression, with collagen type IV and type VII α1 chains showing significant increases. His-tagged 511E8 was stained around the basal cells of HFK layers, not in basal regions. Co-staining with anti-His-tag and anti-integrin β1 antibodies revealed the co-localization of theses in some intercellular regions among basal cells.
Conclusion: These results suggest that 511E8 effected on HFKs, enhancing the production of BM components and strengthening the anchoring between the Col-I gels and the HFK layers.

1. Introduction

Laminin (LM) is a heterotrimeric protein consisting of α-, β-, and γ-chains that forms a superfamily and influences cell behavior such as epithelial cell adhesion and proliferation. In human, five types of α-chains (α1-α5), three β-chains (β1-β3), and three γ-chains (γ1-γ3) have been reported. More than 12 LM isoforms have been identified by combining them, and their tissue localization has been reported [1]. Laminin 332 (LM332), is composed of α3, β3, and γ2

Abbreviations: BM, basement membrane; Col-I, type I collagen; Col-III, type III collagen; Col-IV, type IV collagen; Col-VII, type VII collagen; Col-XII, type XII collagen; Col-XVII, type XVII collagen; DAPI 4′, 6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; FACIT, fibril associated collagen with interrupted triple helices; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; HFFs, human foreskin fibroblasts; HFKs, human foreskin keratinocytes; iPS, induced Pluripotent Stem; LM, laminin; PBS (−), phosphate buffered saline without calcium and magnesium; qPCR, quantitative polymerase chain reaction; SE, skin equivalent; TEM, transmission electron microscopy; 511E8, E8 region fragment of LM511.

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chains, and is a major component of the lamina densa in human skin basement membrane (BM). LM332 has a high affinity for integrin α6β4, which constitutes hemidesmosomes. Hemidesmosomes are dense cytoplasmic plaques that connect epidermal keratinocytes to the BM. The interaction between LM332 and integrin α6β4 is closely related to hemidesmosome formation, stabilization in skin, and maintenance of epithelial cell adhesion. LM332 also correlates with integrin α3β1 to form a focal contact [1,2]. LM511 is the other LM isoform in skin that is composed of LM α5, β1, and γ1 chains. Integrins that bind to LM511 are α3β1, α6β1, and α6β4 [3]. LM511 is present ubiquitously during development and in adults, such as blood vessels, kidneys, lungs, and skin. LM511 has been reported to promote proliferation of several types of cells in experimental systems [1,3]. Under normal resting conditions, human keratinocytes express integrins α2β1, α3β1, and α6β4 [2,4,5], and adhesion to LM511 promotes migration and proliferation of keratinocytes [6]. So pluripotent stem cells such as embryonic stem and induced pluripotent stem (iPS) cells express high levels of integrin α6β1, using LM511 as a culture substrate improves adhesion and allows for efficient culture of undifferentiated pluripotent stem cells [7]. LM511 also promotes cancer cell migration and invasion in experimental systems, suggesting a correlation with malignant transformation of cancer [8]. The LM superfamily influences many physiologically important aspects such as cell adhesion, organogenesis, neural network regeneration, angiogenesis, wound healing, and cancer growth and metastasis. LM molecules have multiple functional domains [1], and their functions have been analyzed using partial laminin fragments [9]. In general, LMs are large molecule, the molecular weight of LM 511 is about 780 kDa, and purification of full-length molecules remains cumbersome and difficult [10]. Previously, the essential constitutive sites of LM511 and LM332 with integrins have been identified. The binding sites are located in the LG1-LG3 domains at the C-terminal of the α chains and the C-terminal end of the γ chains [11,12]. The recombinant relevant E8 region of LM511 (511E8) with a molecular weight of approximately 150 kDa protein is used as a culture substrate for embryonic stem and iPS cells [10,13]. It has also been reported that 511E8 recapitulates to some extent physiological cell–cell and cell–matrix interactions in the native niche environment, enabling the culture of cells such as human limbal melanocytes, which are difficult to culture [14]. Because 511E8 has the unique feature that it is effective for iPS cell culture not only when applied to culture dishes before cell seeding (coating method), but also when added to the culture medium at the time of cell seeding [15], it is considered to have new experimental methods. Since 511E8 also affects cells other than pluripotent stem cells, such as cancer cells [16] and muscle satellite cells [17], it is possible that other cells may also be affected, but its effect on skin cells is still unknown.

The skin is composed of subcutaneous tissue, dermis and epidermis, forming a layer structure with different properties. The dermis and epidermis are anchored by the BM structure. The epidermis is composed primarily of epidermal keratinocytes. Keratinocytes which in contact with the BM (basal cells) proliferate, and cells that leave from the BM differentiate, forming layers of cells with varying degrees of differentiation, eventually dying and shedding from the body surface [18]. One of the important functions of the BM is to precisely connect the dermis and epidermis, and failure of the connection could result in serious disease [19]. There is a thin sheet-like structure called lamina densa, which is composed of type IV collagen (Col-IV), LM, perlecan, nidogen, and other ECM components assembly between the basal cells and the dermal connective tissue. Lamina densa and basal cells are connected by hemidesmosome, and the lamina densa and dermis are connected by anchoring fibrils. Anchoring fibrils are composed of type VII collagen (Col-VII) [20–22].

Skin equivalent (SE) is being studied as an alternative to animal experiments and as a skin substitute for transplantation therapy. There are various SE preparation methods, one of which is to use a contracted collagen gels which made of type I collagen (Col-I) containing fibroblasts as a dermal equivalents and seed keratinocytes on gel surface [23,24]. Many studies have been conducted by applying or modifying this method. In these SEs, as in skin, keratinocytes proliferate and form a cell layer with different degrees of differentiation. To connect these two different structures, dermal equivalent and keratinocyte layer, a BM-like structure is formed between the Col-I gels and the keratinocyte layer using proteins which produced by the cells. In these types of SE, the test substance can be added during the preparation process to study the effect on SE formation [25–27]. For example, the addition of Col-IV aggregates during the SE preparation process promotes Col-IV accumulation at BM-like structure, and facilitates the growth of human epidermal keratinocytes [25]. There are several reports that the addition of full-length LM or LM fragments promotes SE formation. LM332 added during SE preparation process promotes BM formation [26,27]. There are also reports that the cell recognition sequence peptide “YIGSR” (tyrosine- isoleucine - glycine - serine - arginine) in the LMβ1 chain improves the formation of SE [28]. As such, it has been suggested that the addition of LM fragments to the SE preparation process may promote BM-like structure formation. Therefore, in this study, 511E8 was added during SE preparation process using human foreskin keratinocytes (HFKs) and human foreskin fibroblasts (HFFs) containing Col-I gels, and the BM-like structures constructed were compared to examine the influence of 511E8.

2. Materials & methods

2.1. Cell culture

Newborn HFKs were purchased from Takara Bio (Shiga, Japan, C-12001). HFKs were grown in HuMedia-KG2 medium (KURABO Industrial Ltd., Osaka, Japan) containing 0.03 mM calcium. Newborn HFFs were obtained from KURABO Industries Ltd. (OSAKA, Japan) and maintained in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (D6046, Sigma–Aldrich Japan Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS) (BioWest, France, S1600-500) and 1% penicillin/streptomycin (Sigma–Aldrich Japan).

2.2. Preparation of human SEs

The preparation protocol of SEs was schematically shown in Fig. 1A. SEs were prepared based on previous papers [25–27] with slight modifications. At first, DMEM (D5523, Merck Sigma–Aldrich Co., Germany) containing 10% FBS, 0.1% acid soluble bovine Col-I (Nippi Inc. Tokyo, Japan, ASC-1-100-20), 250 μmol/L L-Ascorbic Acid 2-Phosphate Trisodium Salt solution (AA2P) (Nacalai Tesque, Kyoto, Japan), and 1 X 10^5 cells/mL HFFs was prepared for dermal equivalents preparation. Thereafter, 9.5 mL of the mixture solution was poured into a 60 mm culture-dish and incubated at 37 °C for 3 days to form dermal equivalents. During incubation periods, the Col-I gel continued to contract. The average of contracted Col-I gel diameters was shown in Fig. 1B. The incubated Col-I gels for 3 days was shown in Fig. 1C. After gels were cultured, 0.4 mL of 1 x 10^6 cells/mL HFKs were seeded on the contracted Col-I gels and cultured for 2 weeks in air-liquid interface culture by using SE culture media M1 and M2. SE culture medium M1 was consisted of a 1:1 mixture of DMEM and EGF-free HuMedia-KG2 including

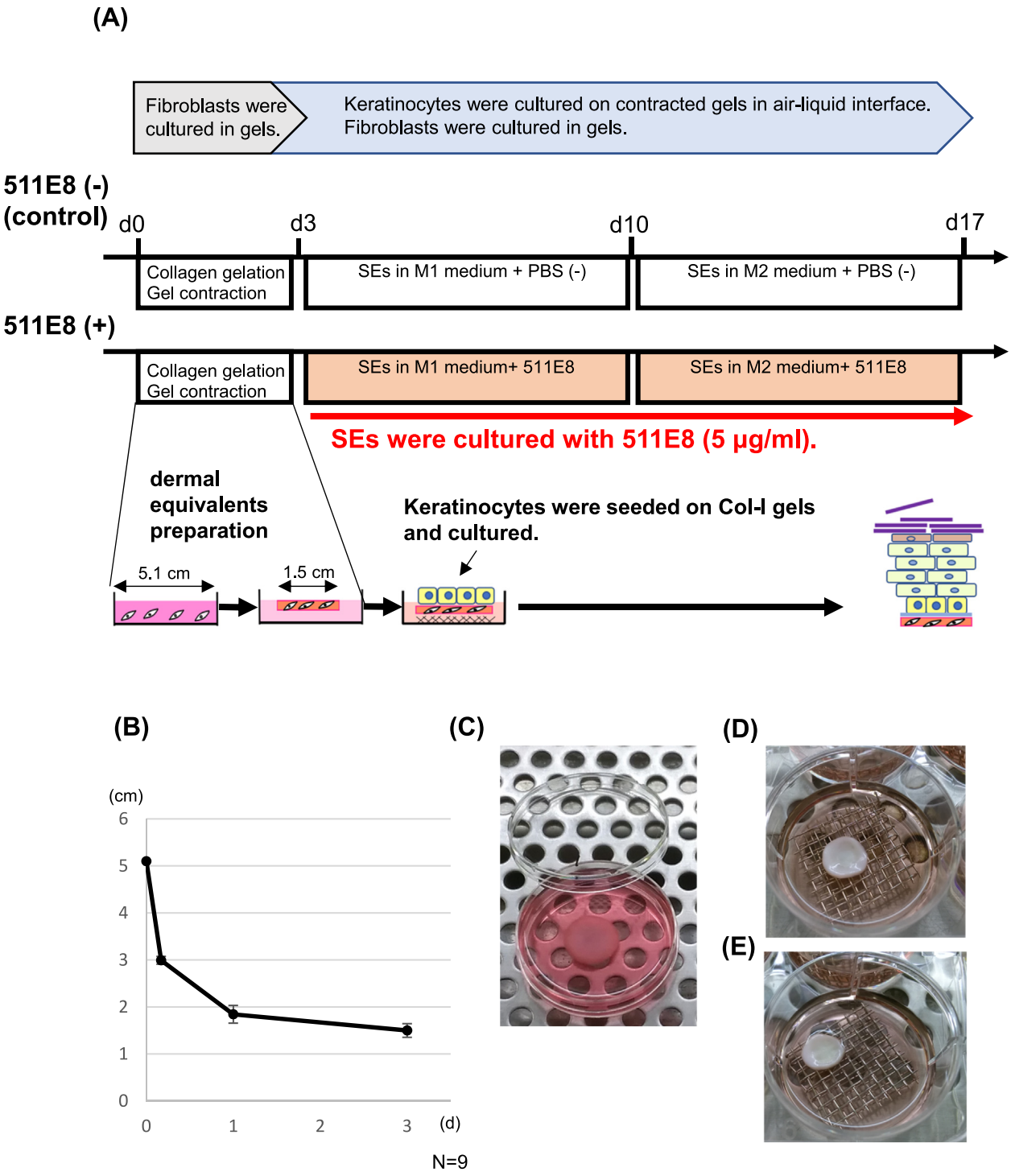

Fig. 1. Preparation process of SEs. (A) Schematic time course of SE preparation protocol. (B) Averages values of the contracted Col-I gel diameter during dermal equivalents preparation. Vertical bars show the standard deviation of the mean (N = 9). (C) Dermal equivalent in day 3. (D) 511E8-non-added SE in day 17. (E) 511E8-added SE in day 17.

1.8 mM calcium, 5% FBS, and 250 μM AA2P. SE culture medium M1 was used from day 3 through day 10. In SE culture medium M2, the protease inhibitors, 10 μmol/l of aprotinin (Merck Sigma–Aldrich, Germany) and 1 μM MMP inhibitor III (Merck Calbiochem) were added in SE culture medium M1 from day 10 through day 17. The medium was changed every 2 or 3 days. In the 511E8-added group, 5 μg/ml of 511E8 (iMatrix-511) (Nippi Inc. Tokyo, Japan) was added to the medium through keratinocyte culture period, and in the 511E8-no-added group (control), the same amount of phosphate-buffered saline (PBS (−); pH 7.4) was added. SEs cultured for 17 days were shown in Fig. 1D (511E8-non-added) and Fig. 1E (511E8-added). At least three SEs per condition were prepared.

2.3. Histological analysis of SEs by using light microscopy

The SEs were pre-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. Subsequently, they were dissected with a razor blade and embedded in paraffin wax in accordance

with the AMeX method [29]. Briefly, small pieces of SEs were dehydrated in an ethanol series and immersed in cold acetone overnight at 4 °C. They were kept in a freezer at −20 °C until they were cleaned in methyl benzoate and xylene consecutively and then embedded in paraffin wax. Paraffin sections at 4 μm were deparaffinized and stained with hematoxylin and eosin (HE) solution.

2.4. Immunohistochemistry analysis of SEs by using light microscopy

To detect BM components, we used mouse anti-LM α3A antibody (BG5; Funakoshi, Tokyo, Japan), rat anti-Col-IV α2 (IV) antibody (C425; Shigei Medical Research Institute, Okayama, Japan) and mouse anti-Col-VII Antibody (clone LH7.2; Sigma–Aldrich, St. Louis, US-MO) respectively for immunohistochemistry. Deparaffinized sections for LM α3 chain were immersed in antigen activation solution (HistoVT One; Nacalai Tesque, Kyoto, Japan) and heated at 90 °C for 30 min. The sections were washed with PBS (−) and treated with methanol containing 0.3% hydrogen peroxidase (H_2O_2) for 20 min at room temperature to remove endogenous peroxidase. The sections were pretreated with 10% rabbit normal serum (SAB-PO(M) Kit; Nichirei Bioscience Inc., Tokyo Japan) at room temperature for 60 min and subsequently incubated with mouse anti-LM α3A antibody diluted 1: 2000 with immune reactivity sensing regent (Can Get Signal immunestain Solution A; TOYOBO, Osaka, Japan) overnight at 4 °C. After being washed with PBS (−), the sections were incubated with rabbit biotinylated anti-mouse IgG + IgA + IgM antibody (SAB-PO(M) Kit; Nichirei Bioscience Inc.) for 30 min and with horseradish peroxidase-conjugated streptavidin-biotin complex (SAB-PO(M) Kit; Nichirei Bioscience Inc.) for 30 min at room temperature. Sections were then incubated with 3,3′-diaminobenzidine terahydrochloride-H_2O_2 solution and lightly stained with hematoxylin. Sections for Col-IV detections using rat anti-Col-IV α2 (IV) antibody diluted 1: 100 without immune reactivity sensing reagent were performed the same manner as above except for the following items, antigen activation treatment was not performed, the goat was used as a normal serum, and goat biotinylated anti-rat IgG antibody (diluted 1: 300; ab6844; abcam, Cambridge, UK) was used as a second antibody. To detect Col-VII, immunohistochemistry staining was performed in the same protocol of using anti-LM α3A antibody. To detect His-tagged 511E8, immunohistochemistry using mouse anti-His antibody (Penta•His Antibody, BSA-free; QIAGEN, Hilden, Germany) diluted 1: 20 without immune reactivity sensing reagent were performed in the same protocol of using anti-LM332 α3A antibody.

2.5. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA from SEs, both in the 511E8-non-added or 511E8-added groups, was isolated by TRIzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration and quality were assessed on a NanoDrop (Thermo Scientific). cDNA synthesis of total RNA was carried out using a High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. Specific primers and probes for real-time qPCR of human Col-I alpha 1 (*COL1A1*), type III collagen (Col-III) alpha 1 (*COL3A1*), Col-IV alpha 1 (*COL4A1*), Col-VII alpha 1 (*COL7A1*), collagen type XII (Col-XII) alpha 1 (*COL12A1*), collagen type XVII (Col-XVII) alpha 1 (*COL17A1*), LM subunit alpha 3 (*LAMA3*), LM subunit alpha 5 (*LAMA5*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were purchased from Thermo Fisher Scientific Inc. (TaqMan® Gene Expression Assays; IDs: Hs00164004_m1, Hs00943809_m1, Hs00266237_m1, Hs00164310_m1, Hs00189184_m1, Hs00990036_m1, Hs00165042_m1, Hs00966585_m1, Hs99999905_m1, respectively). The reaction solution for PCR was prepared in volumes of 20 μl/well containing 10 μl TaqPath™ qPCR Master Mix, CG (Thermo Fisher Scientific Inc.), 1 μl primers and probe, and 9 μl cDNA in RNase-free water (200-fold dilution of cDNA synthesis solution). PCR was performed using two wells per sample on a 7300 Real-time PCR System (Thermo Fisher Scientific Inc.). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C and 45 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C. Standard curves were generated by serial dilutions of the cDNA sample mixture. The Ct value obtained by amplification of each analysis target gene was normalized using *GAPDH* expression level, and then the expression of each gene was compared between the two groups. Statistical differences between the two groups were assessed by Student's t-test. $P < 0.05$ was considered to indicate significance. Values are represented as the mean ± standard deviation in the same manner as previously described [30].

2.6. Histological analysis of SEs by using transmission electron microscopy (TEM) analysis

The SE samples were cut into 2 mm × 1 mm × 1 mm blocks, then immediately fixed by immersion in a half-Karnovsky solution (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) at 4 °C overnight. Tissue membrane structural contrast was enhanced for electron microscopy via heavy metal block staining, as previously described [31]. Briefly, the fixed samples were washed four times with 0.1 M cacodylate buffer (4 min each), immersed in 2% OsO_4 (TAAB Laboratories Equipment Ltd., Berks, UK) and 1.5% potassium ferrocyanide trihydrate (Nacalai Tesque Inc.) in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4 °C; washed four times with distilled water (4 min each); immersed in 0.1% thiocarbohydrazide (Sigma Aldrich, Tokyo, Japan) for 20 min at room temperature; washed four times with distilled water (4 min each); immersed in 2% OsO_4 for 30 min at room temperature; washed four times with distilled water (4 min each); immersed in 1% uranyl acetate at 4 °C overnight; washed four times with distilled water (4 min each); and immersed in Walton's lead aspartate solution at 60 °C for 30 min. Subsequently, the tissues were dehydrated in an ethanol series, transferred to QY-1, and embedded in epoxy resin (Quetol 812; Nisshin EM). After polymerization, the tissue blocks were sliced into 80-nm-thick sections using an ultramicrotome (EM UC7; Leica). The sections were collected on a single-hole grid with Formvar support membrane and observed using a TEM (HT-7700; Hitachi High Technology, Tokyo, Japan) at an acceleration voltage of 80 kV.

2.7. Immunocytochemistry analysis of SEs by using TEM

Immunocytochemistry was performed to observe the localization of 511E8 on an electron microscopic scale. The sample added 511E8 fixed by 4% paraformaldehyde was immersed in 10%, 20%, and 30% sucrose solution at 4 °C for 1 day each, embedded in optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and frozen at −80 °C. Sections of 10 μm thickness were prepared from the frozen sample using a cryostat, and immunocytochemistry was performed in accordance with the following procedures. Sections incubated with mouse anti-His antibody (Penta• His Antibody, BSA-free; QIAGEN, Hilden, Germany) were treated in the same way as immunofluorescence microscopy. After being washed with PBS (−), the sections were incubated with colloidal gold-conjugated goat anti-mouse IgG antibody (diluted 1: 30; Nanogold; 1.4 nm; Nanoprobes Inc., Yaphank, NY, USA) overnight at 4 °C, fixed with 3% glutaraldehyde

for 10 min, and immersed in 0.1 M acetic acid buffer (pH 7.0) for 10 min. Silver enhancement was performed by use of a silver enhancement kit (HQ Silver; Nanoprobes Inc.) in a dark room for 8 min. The sections that showed a positive reaction to immune staining were embedded in a slightly modified manner to the electron microscopy described above, sliced, and observed using a TEM.

2.8. Double immunofluorescence staining for 511E8 and integrin β1

Double immunofluorescence staining was performed to analyze colocalization for 511E8 and integrin β1, a cell surface protein that interacts with 511E8. Deparaffinized sections were pretreated with a blocking reagent (Blocking One Histo; Nacalai Tesque) for 10 min at room temperature and washed with 0.1% Triton X-loaded PBS (−) for 5 min. The sections were incubated with mouse anti-His antibody (Penta•His Antibody, BSA-free; QIAGEN, Hilden, Germany) diluted 1: 20 with PBS (−) overnight at 4 °C. After being washed with PBS (−), the sections were incubated with CF488-conjugated goat anti-mouse IgG serum (diluted 1: 300; Biotium, Hayward, Calif., USA) for 3 h in a dark chamber. After further washed with PBS (−), the sections were incubated with rabbit anti-integrin β1 antibody (diluted 1: 400; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C followed by CF594-conjugated goat anti-rabbit IgG serum (diluted 1: 300; Biotium) for 3 h in a dark chamber. Following additional washed with PBS (−) and being coverslipped with aqueous mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Fluoro-KEEPER; Nacalai Tesque Inc.); finally, they were observed on a fluorescence microscope (ECLIPSE Ni-U; Nikon, Tokyo, Japan).

3. Results

3.1. Morphology with optical microscope

The morphology of the SEs in the 511E8-added and non-added groups were compared with by using HE staining and immunohistochemistry methods. Previous studies [25–27,29,32] using optical microscopy have reported the formation of cell layers with different degrees of differentiated HFKs similar to living organisms. The accumulation of BM components between the cell layer and the contractile gels. In this study, morphological observation of SEs with HE staining showed the formation of HFK layers on contractile gels regardless of the addition of 511E8. No significant differences were observed with (Fig. 2B) or without 511E8 (Fig. 2A). In the 511E8-non-added group, observations with immunohistochemistry methods showed that, as in previous studies of SE, accumulation of BM components, LM α3 chain (Fig. 2C), Col-IV (Fig. 2E), and Col-VII (Fig.G) were observed between the Col-I gels and the keratinocyte basal cells. In this paper, we will refer to cells that contact with the Col-I gels via BM-like structure “basal cells”.

Even in the 511E8- added group, LM α3 chain (Fig. 2D), Col-IV (Fig. 2F) and Col-VII (Fig. 2H) were observed. Although not quantified, there was a stronger and more continuous positive response for LM α3, Col-IV and Col-VII in the 511E8-added group (Fig. 2D, F, 2H) compared to the no-added group (Fig. 2C, E, 2G) respectively.

3.2. Gene expression analysis by qPCR

Gene expression levels of LM α3 chain, LM α5 chain, and the α1 chains of types I, III, IV, VII, XII, and XVII collagens in the SEs at both 511E8-added and non-added group were estimated by qPCR. LM α3, LM α5 chain and Col-IV α1 chain are BM components. Col-I and Col-III are connective tissue components. Col-XII is a member of fibril associated collagen with interrupted triple helices (FACIT) collagens. FACIT collagens bind to the surface of Col-I fibrils, regulate the suplamolecular structure of Col-I fibrils [32], and maintains skin homeostasis [33]. The anchoring fibril component Col-VII is involved in the association of lamina densa to dermal tissue, furthermore, the anchoring filament component Col-XVII is involved in the interaction of lamina densa to basal cells [18,20]. Here, the internal standard was GAPDH, and gene expression levels per cell were estimated and compared 511E8-added and non-added group. Fig. 3 shows the gene expression levels of the 511E8-added sample (black bars) were graphed with respect to the 511E8-non-added sample (grey bars). The gene expression levels of all ECM components examined were slightly enhanced. The addition of 511E8 significantly increased gene expression levels in Col-IV α1 chain and Col-VII α1 chain by Student's t-test analysis.

3.3. Morphology of SEs by TEM observation

TEM observation was performed to confirm in more details of the boundary regions between the Col-I gels and basal cells in 511E8-added and non-added SEs. Formation of BM-like structures was observed in both samples. Comparing 511E8-non-added group (Fig. 4A) and added group (Fig. 4B), more areas of higher electron density regions which indicated arrows in Fig. 4 were observed at the boundaries of SEs in the 511E8-added group, and they were observed more continuous than in the non-added group.

3.4. Localization of 511E8 using by anti-His-tag antibody

We also tried to find out where 511E8 works, but we could not find any effective antibodies for 511E8. So, immunohistochemical staining with anti-His-tag antibody was performed to confirm the localization of the added 511E8 in SEs. Because 511E8 is His-tagged in the purification process [11], the localization could be detected by using anti-poly-histidine antibody. First, an optical microscopic observation was performed of the SEs stained with anti-HIs-tag antibody. No staining was observed around basal cells in the 511E8-non-added sample (Fig. 5A). Arrows indicate Col-I gel and basal cells boundary. In the 511E8-added group, staining was observed in the intercellular regions of basal cells. While, boundary regions between the Col-I gels and the basal cells were not stained (Fig. 5B arrows). The flat cells' periphery on the apical side of the basal cells was also stained (Fig. 5B arrow heads). For more detailed observations, immunohistological TEM observation around basal cells in the 511E8-added SEs was performed by using a gold colloid-labeled antibody. Low magnificated observation showed that the gold colloids were concentrated around the cells except for the boundary regions between gels and basal cells as in the case of optical observation (Fig. 5C). Fig. 5C’ shows a schematic positional relationship of basal cells in Fig. 5C. Fig. 5D is the higher magnification of the inset rectangular region in Fig. 5C. The intercellular regions, intricate protrusions were observed between cell–cell regions of the basal cells, and accumulations of gold colloids were observed around the protrusions (Fig. 5D arrows).

3.5. Co-localization of 511E8 and integrin β1

We tried to confirm the interaction between 511E8 and cell surface integrins in the intercellular regions of basal cells. For this purpose, we used immunofluorescence method to co-stain His-tag detected with CF488-conjugated antibody and integrin β1 detected with CF594-conjugated antibody. In the non-added group, His-tag did not show positive staining in the intercellular regions of basal cells (Fig. 6A). The positive staining around the flat cells on the apical side of the basal cells was observed (Fig. 6A arrow heads) as in the case of immunohistochemical staining in the 511E8-added SE

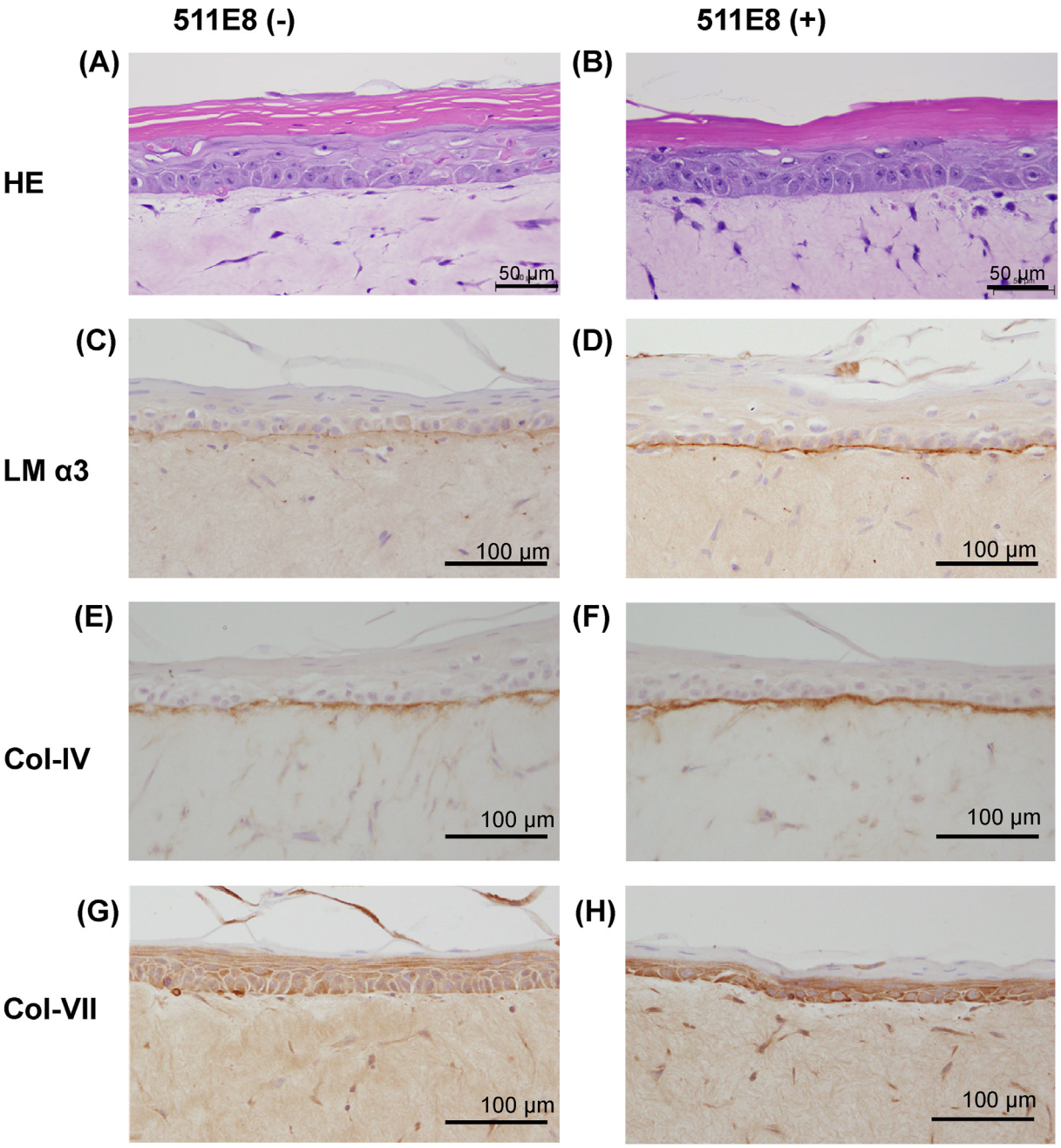

Fig. 2. Histological analysis of SEs with optical microscope. SEs of the 511E8-non-added group (A, C, E, G) and the 511E8-added group (B, D, F, H) were observed by using an optical microscope. The samples were stained with HE solution (A, B), anti-LM α3 antibody (C, D), anti-Col-IV antibody (E, F), and anti-Col-VII antibody (G, H). Scale bars indicate 50 μm (A, B) and 100 μm (C-H).

(Fig. 5B). While in the 511E8-added group, His-tag showed positive staining in the intercellular regions of basal cells without gel boundary region (Fig. 6D). The periphery of the flat cells on the apical side were stained, too (Fig. 6D arrow heads). Integrin β1 showed a positive staining around basal cells including the BM-like structure boundary regions, independent of the addition of 511E8 (Fig. 6B–E). Co-localization of His-tag and integrin β1 was not observed in the non-added group (Fig. 6C). In the 511E8-added group, co-localization of His-tag and integrin β1 was observed at cell–cell interaction regions (Fig. 6F, arrows), but no co-staining was observed in the BM-like structural regions between the gel and the basal cells (Fig. 6F).

4. Discussion

LM511 is thought to correlate with antiaging and plays functional roles for epidermal homeostasis in skin. For instance, LM511 is reported to promote keratinocyte migration and proliferation via binding to α3β1 integrin [3,6]. LM511 is also important for hair morphogenesis and growth to maintain hair follicle stem cells [3]. Furthermore, decrease of LM511 in BM reduces epidermal stem/progenitor cells in skin [34]. As above, although there have been several reports on the effects of full-length LM511 on skin cells, little is known about the effects of LM fragment 511E8 on skin cells. In this paper, we added 511E8 in the culture medium during SE

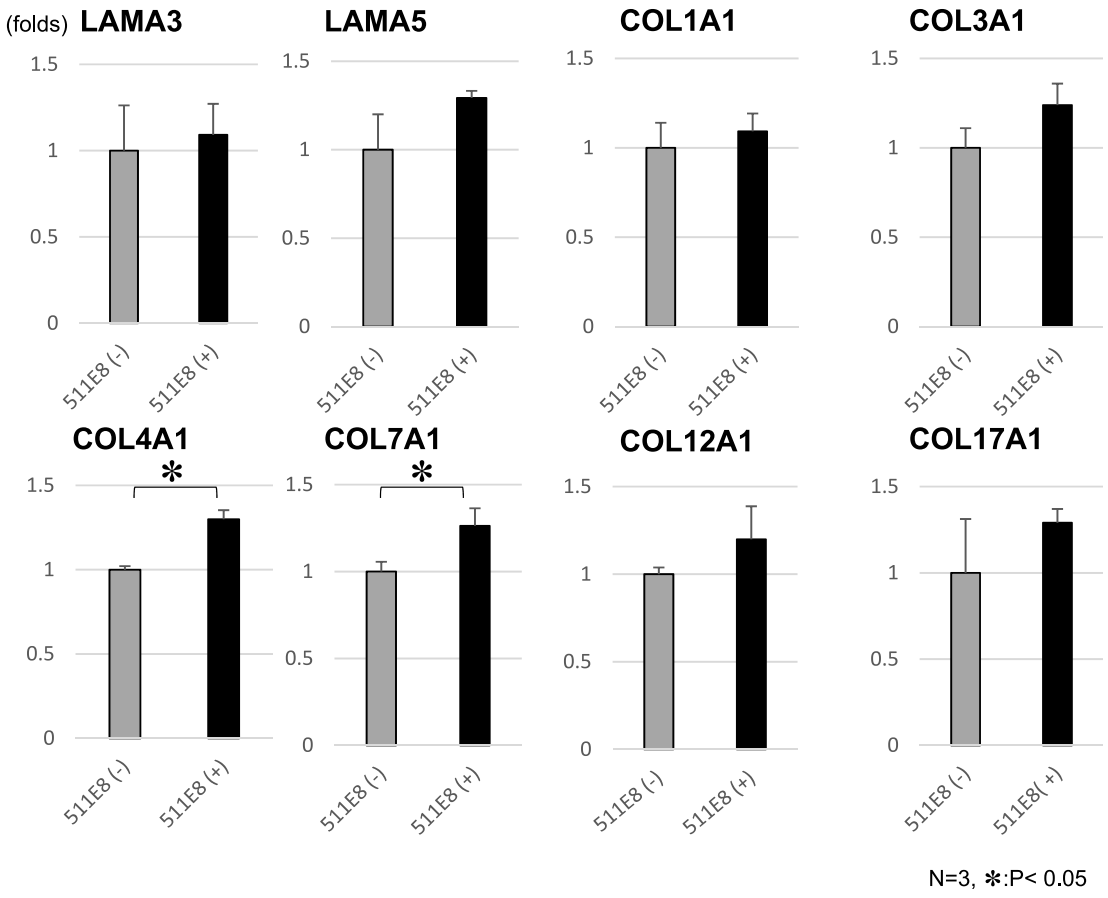

Fig. 3. Quantitative analyses of gene expression levels of LM α3, α5 chains and type I, III, IV, VII, XII, and XVII collagen α1chains in SEs. Total RNAs were prepared from each SE and gene expression levels were measured by qPCR as described in the text. Averages of the gene expression levels in the 511E8-added samples (black bars) were graphed with respect to the 511E8-non-added samples (grey bars). The experiments were performed in triplicate, and data are shown as means ± SD. Differences between two individual groups are analyzed using Student's t-test. *$P < 0.05$.

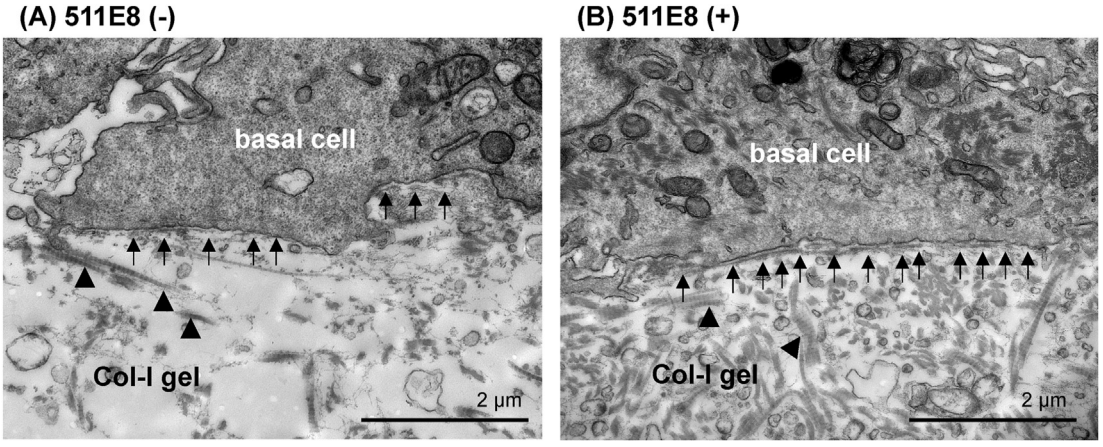

Fig. 4. Ultrastructural analyses for the formation of BM-like structure between Col-I gels and basal cells in SEs. SEs were cultured in the absence (A) or in the presence (B) of 511E8 and then processed for TEM observation as described in the text. In both samples, High electron density regions (arrows) were indicated between Col-I gel and keratinocyte basal cells boundary regions. Arrowheads indicate Col-I fibrils. Scale bars indicate 2 μm.

preparation process to study the effect of 511E8 on skin cells and compared the formation of BM-like structures. Then, the addition of 511E8 enhanced the production of BM component genes per cell (Fig. 3), and morphological observation showed that the Col-I gels and keratinocyte layers were more tightly connected than the 511E8-non-added case (Fig. 4). On the other hands, the expression of stem cell marker p75NTR (CD271) was not affected. Gene expression levels of p75NTR were very low in this experimental system regardless of 511E8 addition. (data not shown). In other words, unlike the papers on full-length LM511 mentioned above

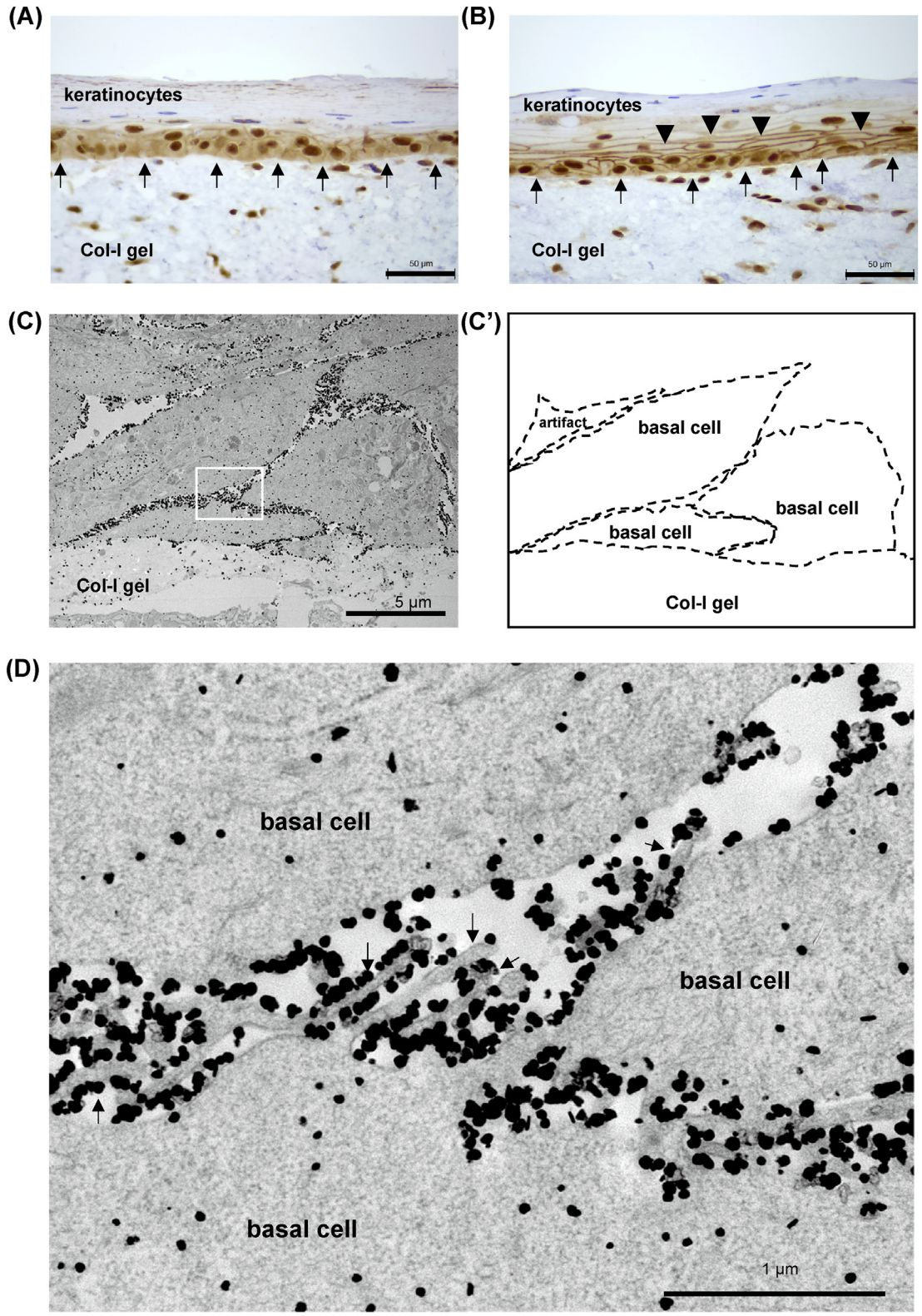

Fig. 5. Localization analysis of the added 511E8 in SEs. Immunohistochemical stained SEs with anti-His-tag antibody in the absence (A) or in the presence of 511E8 (B) were observed by using optical microscope. Scale bars indicate 50 μm. Arrows indicate the boundary regions between Col-I gels and basal cells. Arrow heads indicate flat cells on the apical side of the basal cells (B). For transmission electron microscopic immunolocalization of poly-histidine around keratinocyte basal cells in 511E8 presence SE, a gold colloid-labeled antibody was used for the secondary antibody as described in the text. The high electron density particles between cells were silver enhanced gold colloids (C, D). Scale bar indicates 5 μm (C). Fig. 5C' shows a schematic positional relationship of basal cells in Fig. 5C. Fig. 5D is the higher magnification of the inset rectangular region in Fig. 5C. Scale bar indicates 1 μm (D).

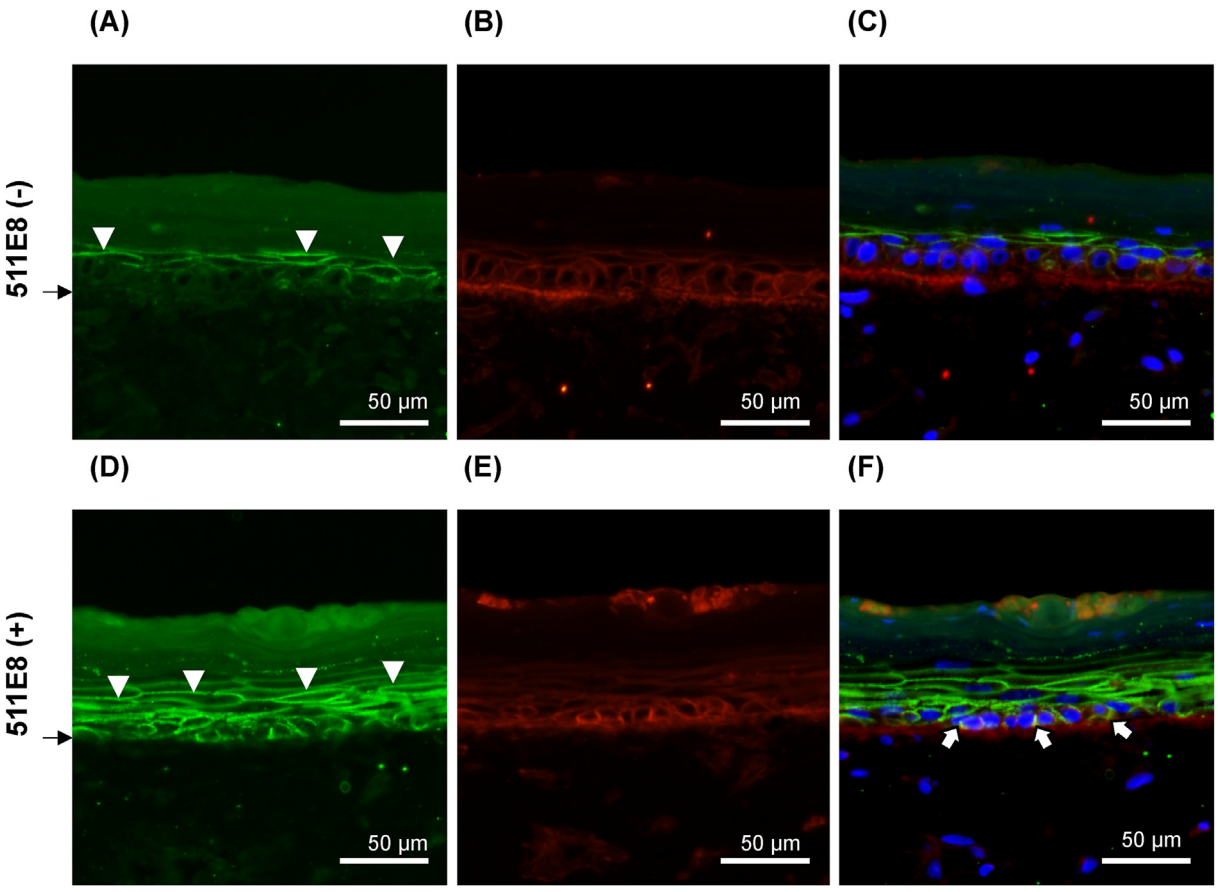

Fig. 6. Co-localization analysis of 511E8 and integrin β1 in SEs. SEs of the 511E8-non-added group (A–C) and the 511E8-added group (D–F) were observed by immunofluorescence staining. After fixed SEs, they were stained with anti-His-tag antibody (A, D) and anti-integrin β1 antibody (B, E), followed by secondary antibodies and DAPI. The merged images are shown on the right (C, F). Black arrows indicate the boundary regions between Col-I gels and basal cells (A, D). White arrowheads indicate flat cells on the apical side of the basal cells. (A, D). White arrows indicate the colocalization of integrin β1 and 511E8 (F). Scale bars indicate 50 μm.

[3,34], in this experimental system 511E8 did not affect the stemness of keratinocytes. So far the cause is unknown, but we think the difference is interesting.

In this study, we used poly-histidine tagged 511E8 and detected by using anti penta•His antibody. In skin, keratinocytes become to express histidine-rich proteins such as filaggrin during differentiation. When Pauly's stain is performed on human skin, intense histidine staining is observed in the granular layer, but not in the basal layer [35]. We speculate one of the reasons of the positive staining around the flat cells on the apical side of basal cells which observed independent of the addition of 511E8 is due to keratinocyte differentiation rather than tag derivation (Figs. 5B–6A and 6D arrow heads). On the other hand, there could be another reason. Comparing Fig. 6A and D, it was clearly stronger stained them in Fig. 6D. This suggests that the added 511E8 might adhere to not only basal cells but also flat cells. We believe that the positive staining of flat cells was occurred from these two reasons.

Previous studies have reported that when BM components are added in SE culture system, the added BM components strengthen the BM-like structure by mechanisms such as (1) being directly incorporated into the BM-like structure [25–27] or (2) acting on the cells to enhance the production of other ECM components and cell growth [27]. The present results indicate that the effect of 511E8 is the "cell-activating" type. BM is a complex structure formed by varieties of proteins. Via specific domains, LMs interact with each other or with other ECM components to form multi-molecular aggregates [1]. Col-IV also self-aggregates and forms fine mesh-work structure [36,37]. During the formation of BM structure, integrins correlate to accumulate LM, Col-IV, nidogen, etc. [38]. In this complex BM formation process, it is unlikely that 511E8, which is composed only of integrin-recognizing sites, binds to integrin and then further interacts with other ECM proteins to be incorporated into BM. Prior to the experiment, we hypothesized that if 511E8 was incorporated into the BM-like structures, it would be randomly trapped into the fine type IV collagen mesh-work. We also thought that such incorporation might instead inhibit signal activation into the cell. The results, however, contradicted expectations, 511E8 was not incorporated into BM-like structures, but could be interacted via integrin β1 in basal cells, upregulating gene expression of some BM components.

Consider upregulation of gene expression and integrin and 511E8 co-localization together, we think that 511E8 which interact with integrin β1 may facilitate signal transduction for BM component synthesis. For the following reason, we believe that investigating the interaction between integrins and 511E8 at cell–cell regions may help elucidate the previously unknown mechanisms involved in basal cell regulation.

In vivo and *in vitro*, integrins α2β1 and α3β1 are known to be present at cell–cell regions in keratinocyte clusters, and their ligands and functions were once controversial with conflicting reports in several groups. Carter et al. suggested the possibility of enhancing integrin-mediated intercellular adhesion [39,40]. While, some researchers believe that integrins play little or no role in intercellular adhesion. They believe that the main regulator of

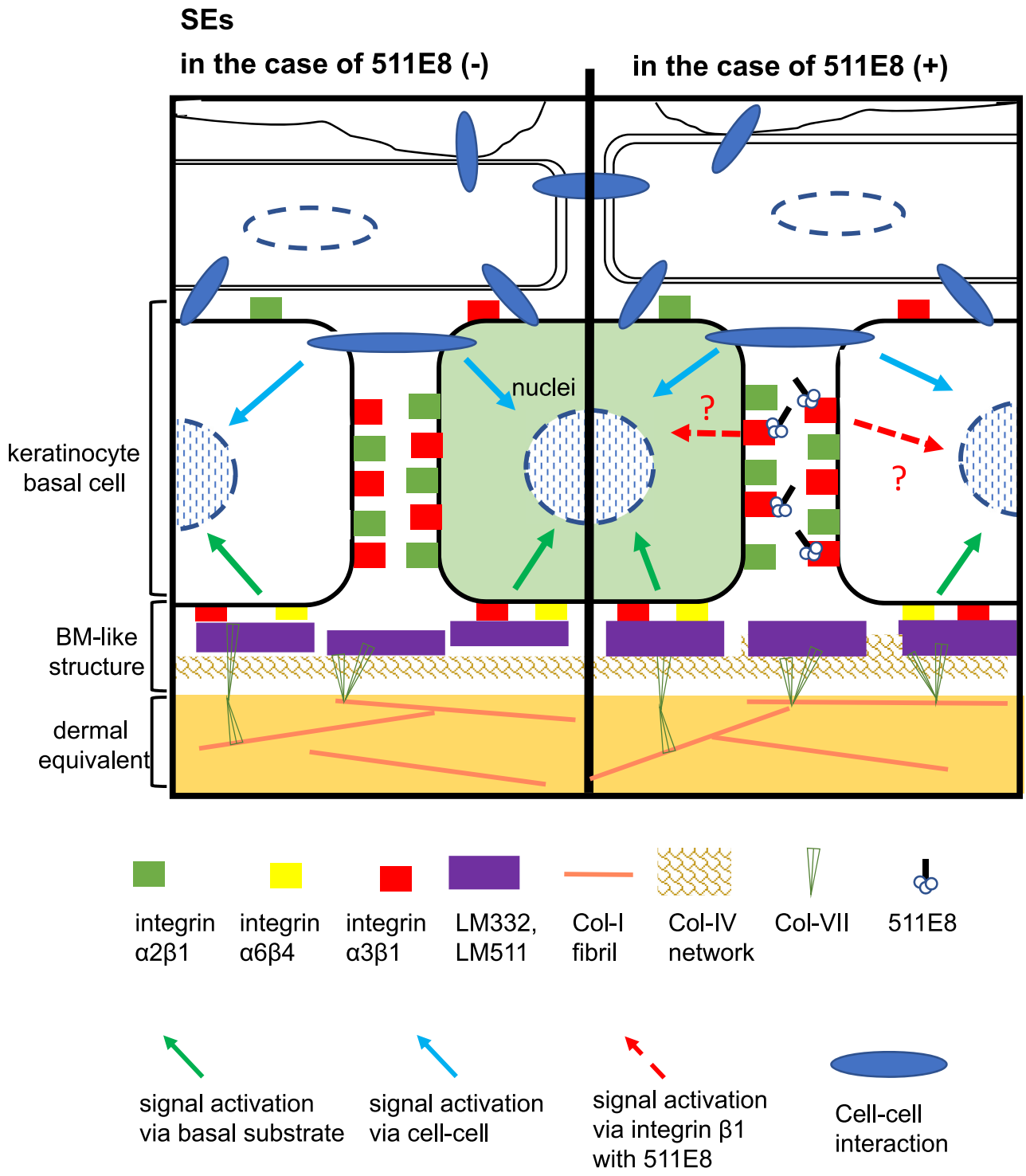

Fig. 7. Scheme of hypothetical effects of 511E8 on SEs. In SE preparation process without 511E8, keratinocytes adhere to dermal equivalent (Col-I gels) to form cell-gel adhesion and cell–cell adhesion. Successively, basal cells proliferate and form differentiated keratinocyte layers, more mature cell-gel adhesion on BM-like structure which cells produced are maintained via integrins α3β1 and α6β4. Cell–cell and cell-gel adhesions activate signal transductions for keratinocyte differentiation, keratinocytes proliferation, and BM components production. Prior studies have shown that integrins α2β1 and α3β1 are present in intercellular of basal cells in skin [40–44] (left panel). Addition of 511E8 during the SEs preparation process, eventually 511E8 may be interacted at the cell–cell regions of basal cells via integrin β1. This interaction of 511E8 to integrin β1 may enhance the signal activation to promote BM components synthesis, in addition to the signals that are activated when 511E8 is not added (right panel).

cell–cell adhesion is not integrins but other intercellular adhesion molecules such as cadherins [41]. Another group reported that inhibitory adhesion experiment with anti-integrin β1 antibody, in keratinocyte intercellular region inhibited cell–cell adhesion in low calcium medium (0.15 mM), but not in high calcium medium (1.2 mM) [42]. They believe that the role of integrins between cells is not definite and that the state of involvement varies depending on conditions. Alternatively, there is a research using specific

antibodies to investigate whether integrin is in a state where it occupies a ligand. Kim et al. report that allow us to infer integrin β1 in the intercellular region is unoccupied state by the ligand [43]. As far as we know, the role of intercellular integrins in keratinocyte remains unclear. The present results showed that the presence of integrin β1 in the intercellular regions around basal cells in SEs, too. And added-511E8 was colocalized to intercellular integrin β1. (Considering integrins α3β1, α6β1, and α6β4 interact with laminins and keratinocytes express integrins α2β1, α3β1, and α6β4 integrin which interacting with 511E8 may be integrin α3β1.) These results, i. e., suggest that intercellular integrins may capture soluble ECM components that does not localize to cell-gel junction and affect basal cells in SEs. A schematic diagram of the hypothesis is shown in Fig. 7. We believe that our results provide the clue to solve new roles for integrins at keratinocyte cell–cell regions. For example, when wound healing, it may be possible to interact the ECM components that are present in body fluids as a liquid factor via integrins at cell–cell regions and utilize them to accelerate ECM production for wound healing. We hope to elucidate the mechanism.

The signal transduction analysis via integrin adhering to 511E8 is not easy because both proliferation and differentiation are occurring simultaneously in keratinocytes during SE preparation period. In addition, because 511E8 was developed as a culture substrate for stem cells, there have been few studies of signaling in skin cells. Unfortunately, we were unable to elucidate the mechanism of signal transduction in this study. However, the study of signal transduction analysis in two-dimensional culture system of LM511 in stem cells may provide a clue to elucidate this issue, even if it is limited. In stem cells, there have been some reports for signal transduction on 511E8. Takagi et al. reported that the differentiation of human embryonic stem cells into keratinocytes is Rho kinase activity-dependent [44]. Yamashita and co-workers showed that yes-associated protein (YAP) activation is responsible for the failure of cartilage formation from human iPS cells on 511E8 [45]. Yuzuriha et al. reported that hematopoietic potential of pluripotent stem cell was regulated through integrin β1-ILK-β-catenin-JUN pathway in human pluripotent stem cells [46]. Full-length LM 511 has other cell recognition sites besides E8 region, the signal activation via adhesion to full-length LM511 may not be identical that to 511E8. Nevertheless, the signal transduction studies of full-length LM511 are informative, because the importance of LM511 in skin cells, especially hair morphogenesis, has been reported [3,47,48]. Thus, there are reports that adhesion to LM511 or 511E8 activates various signaling pathways in cells. We believe that these reports support our hypothesis.

5. Conclusion

We studied the effect of 511E8 on BM-like structure formation in SEs. Addition of 511E8 in SEs preparation process increased gene expression of some BM components and strengthened the BM-like structure in SEs. On this occasion, 511E8 localized at intercellular regions of basal cells and integrin β1 was colocalized. This suggests that the possibility that signal transduction promoted via intercellular integrin β1 with 511E8 in SE preparation process. We believe that the addition of 511E8 to culture medium as a liquid factor can be applied to other organoids producing methods too and is a useful new method in the field of regenerative therapy.

Statement

During the preparation of this work the authors used DeepL in order to translate a Japanese manuscript into English. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.014.

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